Advances in functional fluorescent and luminescent probes for imaging intracellular small-molecule reactive species

Shiguo Wang, Na Li, Wei Pan, Bo Tang

We summarize recent progress in imaging intracellular small-molecule reactive species (ISMRS) by functional probes. In molecular imaging, functional fluorescent and luminescent probes (e.g., ratiometric, targetable fluorescent, reversible fluorescent, and multi-functional) and corresponding nanoprobes have great potential for investigating ISMRS-mediated cell-signal transduction. We describe design strategies for the development of functional probes. Future research on ISMRS will benefit from recent advances in the development of new functional probes for selective detection of ISMRS.

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Keywords: Intracellular small-molecule reactive species (ISMRS); Fluorescent probe; Luminescent probe; Molecular imaging; Multi-functional probe; Nanoprobe; Ratiometric probe; Reversible probe; Targetable probe

1. Introduction

Reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive chloride species (RCS), reactive sulfur species (RSS), important cations, anions and amino acids belong to the family of intracellular small-molecule reactive species (ISMRS), which are important small signaling molecules in physiological and pathological cellular events that govern cell functions [1–3].

The ROS family includes the superoxide anion radical (O$_2^-$), the hydroxyl radical (·OH), hydrogen peroxide (H$_2$O$_2$), ozone (O$_3$), singlet oxygen (¹O$_2$) and the peroxo radical (ROO$^\cdot$) [4]. ROS are mainly produced by leaking of electrons from the mitochondrial electron transport chain (ETC) [4]. By reduction of oxygen transferred on ETC, NADPH catalyzes the production of a large variety of reactive metabolites (e.g., the RCS family mediated by myeloperoxidase (MPO)-H$_2$O$_2$-Cl$^-$ system and the RNS family mediated by NO) [5–7]. Overproduction and accumulation of oxidizing metabolites (e.g., ROS, RNS and RCS) could induce oxidizing or nitrosative stress in living cells, which has been reported to be harmful to the tissue [4–7]. However, living cells have evolved elaborate mechanisms to maintain equilibrium of the reduction–oxidation (redox) state by controlling the precise balance between the levels of oxidizing metabolites and reducing equivalents [e.g., ascorbate, thiols (RSH), ubiquinol, tocopherol and organoselenium]. RSH are prominent members of RSS that control redox homeostasis mainly through the thiol-disulfide interchange reaction, which has been implicated in many cellular processes (e.g., cell division and differentiation) [8].

To understand fully biological roles of ISMRS in living systems, it is crucial to monitor accurately specific ISMRS at the cell, tissue and organism level. To this end, fluorescent and luminescent probes have become widely employed tools for realtime, non-invasive, sensitive and selective imaging of dynamic changes of ISMRS [9–12]. Conventional fluorescent probes with high signal-to-noise ratio, high quantum yield, membrane permeability and in vivo stability could give selective response to specific ISMRS and transduce changes of
ISMRS levels into a change in fluorescence intensity [13]. However, they merely display single-emission patterns or detect the overall levels of ISMRS without distinguishing different concentrations of ISMRS from different organelles (e.g., mitochondria, nucleus, endoplasmic reticulum and lysosome) when responding to oxidizing or nitrosative stress. Each organelle has evolved a unique mechanism to deal with redox homeostasis, during which the function of each particular metabolite of ISMRS may vary.

Emerging studies in molecular imaging suggest that ISMRS have a wide range of physiological effects on the cell network, depending on the specific identity of the ISMRS, the concentration of the ISMRS, the timing and the sub-cellular localization of their production [4,14–17]. Considering the complex biological environment, every particular metabolite of ISMRS needs to be monitored directly near the sites of production and action (in situ). Conventional fluorescent probes could not meet the needs of researchers. Functional fluorescent and luminescent probes, including ratiometric fluorescent and luminescent probes, organelle-targetable fluorescent probes, reversible fluorescent probes, multi-functional probes and corresponding nanoprobes substantially improve the performance of imaging probes and provide a new avenue to study ISMRS. For instance, organelle-targetable fluorescent probes could localize and accumulate within different organelles for a long time, so improving on the sensitivity of traditional fluorescent probes.

There is a growing interest in designing functional probes (Table 1) to study ISMRS at the sub-cellular level. Lippard brought the biochemistry of mobile Zn$^{2+}$ and NO based on functional fluorescent probes to a new place [15]. Chang took the interplay between H$_2$O$_2$ and NOx a step further by employing functional small-molecule fluorescent probes based on a prominent bioorthogonal reaction (boronate oxidation) [16]. By utilizing targetable fluorescent probes, Murphy explored the elaborate mechanism of the mitochondrial function and ROS [17]. Our group also developed a series of functional probes for bioimaging ISMRS [18,19].

In this article, we discuss ratiometric, reversible, organelle-targetable and multi-functional approaches in designing probes so as to understand further the multiple biological roles of ISMRS.

<table>
<thead>
<tr>
<th>Year</th>
<th>Milestone</th>
<th>Primary group leaders</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1986</td>
<td>The first ratiometric and cell-trappable fluorescent probe for Ca$^{2+}$ that laid a solid foundation for designing ratiometric and cell-trappable fluorescent probes</td>
<td>Roger Y. Tsien</td>
<td>[38,85]</td>
</tr>
<tr>
<td>2002</td>
<td>The first ratiometric fluorescent probe for Zn$^{2+}$</td>
<td>K.R. Gee</td>
<td>[39]</td>
</tr>
<tr>
<td>2006</td>
<td>The first ratiometric fluorescent probe for H$_2$O$_2$</td>
<td>Christopher J. Chang</td>
<td>[52]</td>
</tr>
<tr>
<td>2009</td>
<td>Increasing the sensitivity of cell-trappable fluorescent probes by ester functionalization</td>
<td>Tetsuo Nagano</td>
<td>[92]</td>
</tr>
<tr>
<td>2010</td>
<td>Cell-trappable probe for intracellular NO detection that brings the biochemistry of mobile Zn$^{2+}$ and NO based on functional fluorescent probes to a new land</td>
<td>Stephen J. Lippard</td>
<td>[15,93,94]</td>
</tr>
<tr>
<td>2010</td>
<td>FRET-based fluorescent probe for (\cdot)OH imaging</td>
<td>Bo Tang</td>
<td>[51]</td>
</tr>
<tr>
<td>2010</td>
<td>The first ratiometric fluorescent probe for Cu$^{2+}$</td>
<td>Christopher J. Chang</td>
<td>[41]</td>
</tr>
<tr>
<td>2011</td>
<td>The first ratiometric fluorescent probes for NO and OCl$^-$</td>
<td>Weiying Lin</td>
<td>[55,59]</td>
</tr>
<tr>
<td>2011</td>
<td>A series of H$_2$O$_2$ responsive probes that extend boronate oxidation as a prominent bioorthogonal reaction to design fluorescent probes</td>
<td>Christopher J. Chang</td>
<td>[16,132]</td>
</tr>
<tr>
<td>2011</td>
<td>Exploring the elaborate mechanism of mitochondrial function and ROS</td>
<td>Michael P. Murphy</td>
<td>[17]</td>
</tr>
<tr>
<td>2011</td>
<td>Ratiometric fluorescent probes for Fe$^{2+}$, ONOO$^-$ and Cl$^-$; reversible fluorescent probes for imaging redox cycles</td>
<td>Bo Tang</td>
<td>[18,19,58,114]</td>
</tr>
<tr>
<td>2012</td>
<td>O$_3$-responsive mitochondrial fluorescent probes</td>
<td>Bo Tang</td>
<td>[77]</td>
</tr>
<tr>
<td>2012</td>
<td>Ratiometric fluorescent probes for Cu$^{2+}$; multi-functional probe for simultaneously imaging H$_2$O$_2$ and NO to provide more precise data for detecting ISMRS</td>
<td>Weiying Lin</td>
<td>[43,121]</td>
</tr>
</tbody>
</table>

ISMRS, Intracellular small-molecule reactive species.
2. Probes for ratiometric imaging of ISMRS

2.1. Design of ratiometric fluorescent and luminescent probes for cell imaging

Conventional fluorescent probes for cell imaging convert intracellular ISMRS levels to switch on or off fluorescence and display merely single-emission patterns after reacting with target-specific ISMRS. However, the fluorescent properties tend to be influenced by several factors under complex cellular conditions [e.g., the probe concentration and changes in cellular microenvironments (e.g., temperature and pH)]. Ratiometric imaging is based on self-calibration of fluorescence intensity through measuring the two distinct signals in the presence or absence of ISMRS, by which changes in concentration of the target molecule can be detected independent of such factors [20]. Thus, ratiometric imaging is a precise, practical means for detecting ISMRS, as it allows for quantitative measurement of ISMRS levels and provides more accurate data, which is essential to address the challenges in elucidating the complex interplay between ISMRS and pathological events.

Most reported promising ratiometric systems are based on ISMRS-induced modulation of fluorescence resonance-energy transfer (FRET), intramolecular charge transfer (ICT) [20] between donor and acceptor, or nano-embedding [incorporation of reference dyes and specific small-molecule fluorescent probes in a single nanoparticle (NP) [21] (Fig. 1)]. Small-molecule dyes {e.g., cyanine [19], rhodamine [22], boron-dipyromethene (BODIPY) [23] and coumarin [24]} are frequently employed as scaffolds for construction of ISMRS-responsive ratiometric probes. The frequently employed fluorescent probe pairs for FRET are summarized in Table 2. These ratiometric systems have been used to construct probes for imaging pH, anions, metal ions and ROS/RCS/RNS/RSS [22–26].

Among them, FRET is a non-radiative energy-transfer process, in which an excited dye donor transfers energy to a dye acceptor. In the presence or absence of ISMRS, imaging based on FRET allows the simultaneous recording of two emission intensities at different wavelengths. To design small-molecule FRET ratiometric fluorescent probes for ratiometric detection of specific ISMRS, it is necessary to formulate a FRET platform, which comprises an energy donor, a linker and an energy acceptor (Fig. 1). An appropriate interaction site for specific ISMRS is then introduced on the FRET platform to modulate the energy-transfer efficiency [20].

For ICT, the most frequently used strategy is to modify the electron-donating group (e.g., 4-amino donor) of fluorophore into a more electron-withdrawing group (often carbamate with ISMRS-responsive moiety), so suppressing an ICT process upon excitation by light can lead to a blue shift in its emission spectrum and result in a ratiometric signal to allow quantitative determination and imaging ISMRS [20,27,28]. The elaborately-designed ISMRS-responsive moiety within the carbamate group could be specifically recognized by ISMRS and in response back to the amine, which would provide a switch for ratiometric detection of the corresponding ISMRS.

It is notable that, by incorporating different hydrophobic dyes and specific small-molecule fluorescent probe in a single NP without complicated synthetic and separating procedures, fluorescent ratiometric nanoparticles with good water dispersivity provide another fascinating and promising approach to construct ratiometric probes in living cells [21]. Nanostructured materials [e.g., polymers, fluorescent silica NPs and up-converting luminescent NPs (UCNPs)] are frequently employed for ratiometric dual-wavelength fluorescent and photoluminescent imaging [29–35]. In this review, we do not detail fluorescent protein probes that require genetic manipulation, which frequently adds complications [36].

2.2. Ratiometric fluorescent probes for cations

The essential activity of cations is indispensable to cellular events [9,37–47] (e.g., Fe^{3+}/Fe^{2+} and Cu^{2+}/Cu^{+} are reported increasingly to be involved in the metabolism of ROS and RNS). Fe^{2+} and Zn^{2+} may also facilitate the generation of ROS or RNS, respectively. Ca^{2+}, Na^{+} and K^{+} are the essential ions that maintain charge balance and electron transfer. These metal ions are key modulators of biochemical-signaling pathways in neurons, so there is a growing interest in designing ratiometric probes for metals, which are receiving greater attention to bridge the gap.

More than a decade ago, Tsien et al. designed the first ratiometric Ca^{2+}-sensitive probe 1a based on the Fura-2 family, which gave selective response to Ca^{2+} over other metal ions [38]. Ten years ago, Gee et al. developed the first ratiometric Zn^{2+}-sensitive probe 1b based on excited-state intramolecular proton transfer (ESIPT) to visualize the movement of Zn^{2+} in cells [39].

Since that time, several metal ion-responsive ratiometric fluorescent probes have been developed [16] (Fig. 2). These probe designs are generally based on FRET, ICT, intracellular ester hydrolysis and nano-embedding. The interesting photochemical properties of selected radiometric fluorescent probes are summarized in Table 3. However, some probes based on the Fura-2 and Indo-1 family require UV excitation, which can be damaging to cells and present problems with autofluorescence when applied in vitro. Among them, the cell-permeable Zn^{2+} ratiometric fluorescent probe 2 designed by Lippard et al. requires close attention [40]. In probe 2, the ratiometric imaging of Zn^{2+} was based on Zn^{2+}-controlled switching between fluorescein and naphthofluorescein tautomeric forms with single-excitation, dual-emission
profiles. Seminaphthofluorescein was chosen as the fluorophore and 2-[bis(2-pyridylmethyl)-aminomethyl]aniline as the receptor of Zn\(^{2+}\). Probe 2 showed excellent selectivity responses for Zn\(^{2+}\) over competing Ca\(^{2+}\) and Mg\(^{2+}\) at intracellular concentrations with dissociation constant (K\(_a\)) for Zn\(^{2+}\) of <1 nM and an 18-fold increase in fluorescence-emission intensity ratio (F\(_{624}/F_{528}\)) upon zinc binding. It could detect endogenous stores of intra-

**Table 2.** Fluorescent probe pairs for FRET

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Donor(D) Wavelength(nm)</th>
<th>Acceptor(A) Wavelength(nm)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>[24,47]</td>
<td>Cy3</td>
<td>Cy5</td>
<td>Cyanine: easy-modification and emission in NIR region in which background autofluorescence can be eliminated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ex:550, Em: 570</td>
<td>Ex: 650, Em: 670</td>
</tr>
<tr>
<td>[24]</td>
<td>BODIPY</td>
<td>Rhodamine</td>
<td>BODIPY: high extinction coefficient, high quantum yields, excitation and emission profiles insensitive to solvent polarity and pH</td>
</tr>
<tr>
<td></td>
<td>Ex485, Em:510</td>
<td>Ex: 550, Em: 589</td>
<td></td>
</tr>
<tr>
<td>[43,55]</td>
<td>Coumarin</td>
<td>Rhodamine</td>
<td>Rhodamine: high absorption coefficient, high fluorescence quantum yield, photostability and broad fluorescence in the visible region</td>
</tr>
<tr>
<td></td>
<td>Ex: 410, Em: 473</td>
<td>Ex:560,Em:583</td>
<td></td>
</tr>
<tr>
<td>[51]</td>
<td>FAM</td>
<td>AuNP (quencher)</td>
<td>Fluorescein dyes: excellent labeling fluorophore, high quantum yields and water solubility;</td>
</tr>
<tr>
<td></td>
<td>Ex: 496, Em: 518</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[52]</td>
<td>Coumarin</td>
<td>Fluorescein</td>
<td>Coumarin: large Stokes shift, easily conjugated to other dyes for efficient energy transfer</td>
</tr>
<tr>
<td></td>
<td>Ex: 420, Em: 464</td>
<td>Ex:461,Em:517</td>
<td></td>
</tr>
<tr>
<td>[63]</td>
<td>Coumarin</td>
<td>Porphyrin</td>
<td>Porphyrin: tunable photophysical properties by modifications on the nucleophilic hydroxyl groups</td>
</tr>
<tr>
<td></td>
<td>Ex: 420, Em: 459</td>
<td>Ex:421, Em:658</td>
<td></td>
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</table>

The term “cyanine dye” denotes a dye system with a polymethine chain between two nitrogens (i.e. R,N-CH—CH-n-CH—N’R”). Cyanine dyes are given common names according to the number of carbon atoms between the dihydroindole units. Cy3: cyanine dye with three carbon atoms between the dihydroindole units; Cy5: cyanine dye with five carbon atoms between the dihydroindole units; BODIPY: boron-dipyromethene; FAM: carboxyfluorescein; AuNPs: gold nanoparticles
cellular Zn$^{2+}$ after NO-induced release of Zn$^{2+}$ from cellular metalloproteins. This connection of Zn$^{2+}$ and NO laid a solid foundation for studies of the connection between Zn$^{2+}$ and RNS.

Cu$^{2+}$/Cu$^{+}$ are involved in bone formation, cellular respiration and connective tissue development. Shifting the Cu$^{2+}$/Cu$^{+}$ redox equilibrium is closely connected with oxidative stress and neurological disorders, including Alzheimer’s, Parkinson’s, Menkes’ and Wilson’s diseases [41]. Thus, ratiometric detection of Cu$^{2+}$/Cu$^{+}$ redox equilibrium is of paramount significance. So far, for specific ratiometric imaging intracellular Cu$^{2+}$/Cu$^{+}$ homeostasis, ratiometric fluorescent probes 3, 4 and 5 have been developed. In 2010, Chang et al. developed ratiometric Cu$^+$ fluorescent probe 3 composed of an asymmetric BODIPY as fluorophore and thiouether-rich receptor as modulator for binding Cu$^+$ [41]. In absence or presence of Cu$^+$, the emission intensity at 505 nm was unchanged but the emission band at 570 nm changed dramatically, which made 3 suitable for ratiometric imaging of intracellular Cu$^+$ homeostasis. Probe 3 showed high selectivity for Cu$^+$ over other metal ions and a ~20-fold fluorescence ratio change with visible excitation and emission profiles.

In 2011, Lu et al. reported the importance of dual-emission fluorescent silica NP-based Cu$^{2+}$ probe 4 that comprised a dye-doped silica core as a reference signal and a Cu$^{2+}$ receptor covalently grafted on the surface of

| Table 3. Photochemical properties of ratiometric fluorescent probes |
|------------------------|-----------------|----------------------|-----------|
| Probes | Wavelength shifts | Comments | Ref. |
| 1a | The binding of Ca$^{2+}$ shifted the fluorescence excitation spectrum of probe to shorter wavelengths at 340–350 nm with a depressed excitation spectrum at 380–390 nm. The emission spectrum peaks at 505–510 nm and hardly shifts wavelength | The first ratiometric fluorescent probe for Ca$^{2+}$ with a selective response to Ca$^{2+}$ over other metal ions | [38] |
| 1b | Fluorescence wavelength shift from 480 nm to 395 nm (excitation at 350 nm) | The Zn$^{2+}$ dissociation constant of the probe is 3.0 μM, showing a high affinity for Zn$^{2+}$ | [39] |
| 3 | Fluorescence wavelength shift from 570 nm to 556 nm with the emission intensity at 505 nm unchanged (excitation at 480 nm) | It showed 20-fold fluorescence ratio change upon Cu$^+$ binding | [41] |
| 6 | The fluorescence intensity at 635 nm was quenched with the emission intensity at 507 nm unchanged (excitation at 485 nm and 569 nm) | Near-infrared fluorescence could reduce the interferences from background fluorescence and light scattering | [19] |
| 8 | With decreasing pH, the ratio of fluorescence intensities emitted from donor and acceptor dyes at two emission wavelengths, 570 nm and 670 nm increased (excitation at 543 nm) | It could discriminate variations in acidity of cellular organelles | [47] |
| 10 | The referenced signal 670 nm/800 nm increases up to 4.5-fold from oxygen-saturated to oxygen-free conditions (excitation at 635 nm) | The nanosensors could be targeted for imaging of hypoxic conditions due to accumulation of the nanosensors in tumor tissue | [48] |
| 11 | The fluorescence intensity ratios of probe at 495 nm and 651 nm showed a dramatic change from 0.018 to 3.8 upon treatment with ‘OH (excitation at 460 nm) | The probe features high selectivity for ‘OH, a very large emission shift (156 nm), a large ratiometric signal (a 210-fold variation), emission in the NIR, high stability in solution, excitation in visible light, cell-membrane permeability | [50] |
| 14 | The ratio of emission intensities (F$_{459}$/F$_{658}$) varies from 0.3 to 3.6 after treatment with H$_2$O$_2$ (excitation at 410 nm) | The observed rate constant for H$_2$O$_2$ deprotection, k$_{obs}$ = 8.8 x 10$^{-7}$/s. The ratiometric emission response of the probe is highly selective for H$_2$O$_2$ over other reactive oxygen species | [36] |
| 17 | The novel ratiometric fluorescent sensor exhibited a very large variation (up to 420-fold) in the fluorescence ratio (F$_{485}$/F$_{475}$) (excitation at 410 nm) | The probe features high sensitivity, high specificity, functioning well at physiological pH, low cytotoxicity, and good cell-membrane permeability | [55] |
| 18 | The nanoprobe gave different emission wavelengths F$_{475}$/F$_{810}$ (excitation at 532 nm and 635 nm, respectively) | The probe has the unique advantages of good water solubility, photostability, biocompatibility, and NIR excitation and emission | [18] |
| 20 | The fluorescence intensity at 436 nm dropped gradually, excited at 318 nm and fluorescence intensity unchanged at 519 nm (excited at 494 nm with increased Cl$^-$ concentration) | The probe is stable with an instantaneous response to fluctuation of Cl$^-$ concentration. It realized the dynamic, visualizable analysis of Cl$^-$-level differences between normal and ischemic ventricular myocytes | [58] |
| 21 | It displayed an 80 nm red shift (excitation at 540 nm) | The probe exhibited high sensitivity and selectivity to OCI$^-$ | [59] |
| 23 | The emission ratio F$_{583}$/F$_{473}$ showed a dramatic increase in the presence of increasing concentrations of Cys (excitation at 350 nm) | It is highly sensitive to thiols with a low detection limit and a large fluorescence dynamic range | [63] |

ISMRS: Intracellular small-molecule reactive species
Figure 2. Structures of ratiometric fluorescent probes 1–8 for imaging cations.
Figure 3. Structures of ROS-activated ratiometric fluorescent probes 9–16.
Figure 3. (continued)
Figure 4. Structures of ratiometric fluorescent and luminescent probes 17–19 for sensing RNS.
Figure 5. Structures and confocal imaging of fluorescent probes 20 and 21 for sensing RCS in living cells. Ratiometric imaging of Cl\(^-\) in ischemic ventricular myocytes (left) and in OCI\(^-\) in live MCF-7 cells (right) incubated with probes.
Figure 6. Structures of RSH-responsive ratiometric fluorescent probes 22–24.
Figure 7. Mechanism of organelle-localized ISMRS fluorescent probes.
Figure 8. Structures of organelle-localized ISMRS fluorescent probes 25–27 based on SNAP tag.

Figure 8. (continued)
the silica NPs. Nanoprobe 4 exhibited a ratiometric fluorescence response to Cu\(^{2+}\) in HeLa cells with limit of detection (LOD) as low as 10 nM [42].

In 2012, ratiometric Cu\(^{2+}\) fluorescent probe 5 was designed on the basis of coumarin-rhodamine scaffold by Lin et al. [43]. FRET is one of the commonly exploited sensing mechanisms for design of ratiometric fluorescent probes. Modulating ratiometric fluorescence of 5 was achieved by FRET from energy donor (coumarin) to acceptor (rhodamine), which led to a large emission-wavelength shift (around 110 nm) between coumarin emission and rhodamine emission. The ratiometric fluorescent probe featured a low LOD (S/N = 3) of 13 nM in HEPES buffer.

Cyanine has found many biological applications in designing probes because of its easy modification and emission in the near-infrared (NIR) region (650–900 nm), in which interference from background auto-fluorescence by intracellular biomolecules can be eliminated [44,45]. However, it is still rare to report constructing ratiometric fluorescent probes based on cyanine.

For ratiometric detecting Fe\(^{2+}\), our group prepared a new NIR fluorescent probe 6 based on cyanine by introducing BODIPY to replacing hydroxyl as the reference fluorophore and the group 4'-[(aminomethylphenyl)-2,2'-6',2''-terpyridine (Tpy) as selectively responsive to Fe\(^{2+}\). This method could be extended to construct other NIR ratiometric fluorescent probes [19]. Ratiometric fluorescent probe 6 showed good selectivity and high sensitivity with the LOD as low as 12 nM upon binding with Fe\(^{2+}\). The probe was stable and could instantaneously respond to Fe\(^{2+}\)-concentration fluctuations with good cell-membrane penetrability and low toxicity.

As minor variations of intracellular pH may induce cellular dysfunction, desirable pH fluorescent probes should be able to respond sensitively to minor changes of pH, and to avoid interference from native cellular species. Imaging and probing the intracellular acidic organelles (e.g., early/late endosomes and lysosomes)
could provide valuable information about disease localization and offer a practical tool for medical therapy and diagnostics. Ratiometric fluorescent probes could probe either molecular interactions or a single molecular event (e.g., detecting acidification in molecular environments). Ratiometric pH probes 7, based on the photoinduced electron-transfer (PET) mechanism, and 8, based on the FRET mechanism, were developed for detecting/imaging intracellular pH changes by Alvarez-Pez et al. [46] and Sung et al. [47], respectively. Ratiometric pH probe 7 was prepared by conjugatively linking the BODIPY fluorophore at the 3-position to pH-sensitive ligand imidazole through an ethenyl bridge [46]. PET occurred efficiently under neutral conditions but inefficiently under acidic conditions. The modest pKₐ value of 4.9 is suitable for detecting changes in the acidic environment in living organisms.

Ratiometric pH nanoprobe 8 was developed based on N-palmitoyl chitosan bearing a donor (Cy3) and an acceptor (Cy5) moiety [47]. At low pH, changes in the environmental pH enabled 8 to gain FRET and more energy transferred from Cy3 to Cy5.

Both 7 and 8 showed the pH-dependent spectral properties that exactly detected H⁺ and afforded more precise data for analysis of roles of H⁺ in ROS metabolism.

2.3. Ratiometric fluorescent and luminescent probes for ROS

In the metabolism of mitochondria, an inadequate oxygen supply could induce hypoxia, which is a feature of many pathological processes (e.g., cancers). Hypoxia is associated with increased metabolic activity and results in an elevated extracellular acidity, so detection and ratiometric imaging of oxygen and hypoxia in solid tumors is of paramount clinical significance for understanding the biological role of oxygen [27,48]. Intracellular fluctuation of local oxygen concentrations can be exactly measured by ratiometric fluorescence intensity.

Recently, for detecting hypoxia, ratiometric fluorescent probe 9 [27] and nanoprobe 10 [48] were developed by Qian et al. and Schäferling et al., respectively (Fig. 3). Probe 9 was developed by connecting a p-nitrobenzyl moiety and signaling moiety 4-amino-N-(2-(2-acetoxethoxy) ethyl) naphthalimide (RHF) via a carbamate group. Upon reduction by oxygen-sensitive nitroreductase (NTR), the amino group of RHF was released to generate the aminonaphthalimide and ICT occurred, resulting in the restoration of fluorescence emission. The maximum emission wavelength of 9 was at 475 nm while RHF was longer by ~75 nm and fell at 550 nm, which made probe 9 favorable for ratiometric imaging of hypoxia.

Luminescent NIR polymer-nanoprobe 10 was developed for in vivo oxygen sensing [48]. Nanoprobe 10 was based on polystyrene NPs (PS-NPs) incorporating an oxygen-sensitive NIR-emissive palladium meso-tetraphenylporphyrin and an inert reference dye, which were both excitable at 635 nm.

Generated by successive monovalent reduction of O₂ in the course of biological metabolism, ‘OH is a very reactive species that has a lifetime of about 2 ns in aqueous solution [49]. The concentration of ‘OH is low in biological systems. Their fluorescence signals suffer from interference by other highly reactive oxygen species (hROS), which are strong oxidants that include ‘OH, hypochlorous acid (HOCI) and peroxynitrite (ONOO⁻). In particular, interference from ONOO⁻ is a severe obstacle in detecting ‘OH because of the similarity between ONOO⁻ and ‘OH with regard to their strong oxidation capacity.

Ratiometric ‘OH fluorescent probes 11 and 12 were developed by Lin et al. [50] and our group [51], respectively. To find applicability in living cells, Lin prepared ratiometric fluorescent probe 11 for ‘OH ratiometric imaging in 2010. Probe 11 was prepared by hybrid coumarin–cyanine dye [50]. In the absence of ‘OH, the free probe displayed an emission band centered at 495 nm. However, upon treatment with ‘OH, there was a marked decrease of emission intensities at 495 nm and concurrently a significant enhancement of emission intensities at 651 nm.

Ratiometric ‘OH fluorescent probe 12 comprised carboxyfluorescein (FAM) as fluorophore and gold NPs (AuNPs) as quencher modules linked by single-stranded DNAs. Based on the hybrid FAM-DNA-AuNP platform, the fluorescence of FAM was quenched. However, ‘OH-induced cleavage of DNA strands would release the free FAM with strong fluorescence [51]. This methodology established the value of this nanotechnology-based probe for imaging ‘OH in living cells and opened a new window to facilitate investigations of ROS-mediated cell behavior.

Chang et al. fully explored an H₂O₂-activatable ratiometric fluorescent probe and devised 13–15 based on boronate oxidation [28,52,53]. Ratiometric probe 13 was designed based on FRET, and comprised a coumarin donor and a boronate-protected fluorescein acceptor linked by a rigid spacer [52]. In the absence of H₂O₂, as a result of suppressed FRET, only blue-donor emission was observed upon excitation of the coumarin chromophore. The FRET-based probe showed a >5-fold higher emission-ratio response to H₂O₂ over other competing ROS (e.g., O₂⁻ or tert-butyl hydroperoxide). Though 13 was successfully applied for selective detection of H₂O₂ in separate mitochondria and capable of quantifying changes in H₂O₂ through a ratiometric fluorescence response, it was not extended to cell imaging. The ratiometric imaging probe 14 was extended to live tissue by tuning the ICT properties through modulating the electron-donating 4-amino donor on 1,8-naphthalimide,
Figure 9. Structures of mitochondria-targeted fluorescent probes 28–35 for detecting ISMRS.
Figure 9. (continued)
Figure 9. (continued)
which affected both ICT and emission color, as making this substituent more electron-deficient resulted in ICT-induced blue shifts in emission maxima [28].

In 2011, two-photon probes 15 [53] and 16 [54] for H₂O₂ were developed for deep tissue imaging. Both 15 and 16 could ratiometrically detect H₂O₂ in live cells and intact tissues at > 100 μm depth through the use of two-photon microscopy (TPM). 16, especially, could monitor changes in mitochondrial H₂O₂, as reported by Kim et al. [54]. TPM provides imaging with increased depth of penetration (4500 mm) and prolonged observation time, due to the use of two NIR photons as the excitation source with less-damaging lower energy light [54].

2.4. Ratiometric fluorescent and luminescent probes for RNS
Nitric oxide (NO) is a diffusible gaseous free radical with an average life-time of ms–s and a large range of biological concentrations (nM–µM), so it tends to form derivatives rapidly in physiological conditions. RNS play a multi-functional role in both physiological and pathological processes [7].

In 2011, Lin et al. developed ratiometric fluorescent NO probe 17 (Fig. 4), which comprised a coumarin donor, a rhodamine acceptor, and a rigid piperazine linker, by taking advantage of the ring-opening process of the rhodamine spirolactam induced by NO, which switches on FRET and brings variations in optical properties [55]. However, in the absence of NO, the rhodamine acceptor was in the closed form, which meant the excitation energy of the coumarin donor could not be transferred to the rhodamine acceptor. Ratiometric fluorescent probe 17 exhibited a highly sensitive “turn-on” fluorescent response toward endogenously-produced NO in macrophage cells. Remarkably, there is a ~420-fold variation in the fluorescence ratio (I₅₈₃/I₄₇₃) from 0.061

Figure 10. Structures and confocal imaging of fluorescent probes 36 and 37 in living cells. Confocal fluorescence image (upper left) and fluorescence lifetime image (upper middle) of fluorescent probe 36 showing a higher temperature in the nucleus (upper right). Confocal imaging of both HEK293 and Hela cells loaded with fluorescent probe 37 and the nuclear stain Hoechst 33342 shows good colocalization of the two dyes, establishing that fluorescent probe 37 accumulates specifically in the nucleus (lower).
in the absence of NO to 26.6 in the presence of 100 equiv. NO.

To solve the selectivity of NIR fluorescent probes towards ONOO⁻ and to monitor accurate fluxes of ONOO⁻, our group developed 18 for highly selectively monitoring the concentration changes of intracellular ONOO⁻ by measuring the ratio of red and green fluorescence intensities [18]. Nanoprobe 18 was based on a polymeric micelle made of poly(d,l-lactic acid) (PLA) and polyethylene glycol (PEG) by incorporating it into rhodamine as reference dye and benzylselenide-tricarbocyanine (BzSe-Cy) as ONOO⁻ indicator, which gave selective response to ONOO⁻. It effectively avoided the influences from enzymatic reaction and high-concentration OH⁻ and OCl⁻.

Ratiometric luminescence probes 19 for detecting and imaging ONOO⁻ in HeLa cells was developed by Yuan et al. [56]. [Eu³⁺/Tb³⁺ (DTTA)] mixture was employed as a ratiometric probe for detecting ONOO⁻. The luminescence of [Tb³⁺ (DTTA)] could be quenched by ONOO⁻ rapidly while [Eu³⁺ (DTTA)] did not respond to ONOO⁻. Luminescent lanthanide complexes have long luminescence lifetimes, large Stokes shifts, and sharp emission profiles; these properties enable them to be used for time-gating. However, ratiometric luminescence probes have absorption in the UV region, which renders them unsuitable for imaging ISMRS.

2.5. Ratiometric fluorescent probes for RCS

RCS mainly include HOCl and Cl⁻. HOCl is a highly reactive species produced from peroxidation of Cl⁻ catalyzed by the enzyme MPO. Mismanagement of them has a close relationship with cardiovascular diseases, myotonia and cystic fibrosis [6,57–59]. Cl⁻ concentration in ventricular myocytes is clinically regarded as a feature of myocardial ischemia, so there is currently great interest in developing ratiometric fluorescent probes that can selectively detect intracellular HOCl and Cl⁻.

In 2011, our group developed ratiometric Cl⁻ fluorescent probe 20 (Fig. 5). 5-Amino-fluorescein (AF) was chosen as reference dye and 6-methoxyquinoline (MQMBP) as Cl⁻ recognition receptor [58]. When the probe was excited using the wavelength of 318 nm (exciting the MQMBP moiety), the fluorescence intensity at 436 nm dropped gradually with the increase of Cl⁻ concentration. Meanwhile, the fluorescence intensity of the AF moiety, as the Cl⁻-insensitive group around 519 nm, kept nearly constant when the probe was excited at 494 nm. Thus, the fluorescence intensity of 20 decreased linearly with the increase of Cl⁻ concentration. Probe 20 showed excellent linearity between fluorescence ratios (F 519/F436) and Cl⁻ concentrations in the range 0.5–100 mM, which correlated well with the Cl⁻ level in ventricular myocytes. The LOD was calculated to be 0.48 mM. These results showed this probe could qualitatively and quantitatively detect Cl⁻ in ventricular myocytes.

For selective detection and imaging of intracellular HOCl, ratiometric fluorescent probe 21 was designed by Lin et al. [59] by taking advantage of the transformation of coumarin-2,4-dinitrophenylhydrazone to coumarin aldehyde promoted by OCl⁻. The intensity of the emission peak of 21 at 585 nm gradually decreased, while the new blue-shifted emission peak centered at 505 nm increased, so 21 could give a ratiometric response to OCl⁻ and was suitable for imaging OCl⁻ in live MCF-7 cells.

As the levels of HOCl are closely connected with homeostasis of pH and ROS, ratiometric fluorescent probes and multi-functional fluorescent probes capable of simultaneously monitoring endogenously-produced ROS and RCS are greatly needed for further studies.

2.6. Ratiometric fluorescent probes for RSS

RSH are important indicators and modulators in intracellular redox homeostasis and cellular growth involved in human health and disease [5,8,60–64]. More precisely, in mitochondria, small-molecular-weight thiols [e.g., cysteine (Cys), homocysteine (Hcy), and glutathione (GSH)] constitute the RSS family, which includes thiols,
Figure 11. Structures of fluorescent probe 38–41.
S-nitrosothiols, sulfenic acids, and sulfite. They control redox homeostasis through the thiol-disulfide interchange reaction, which has been implicated in many cellular processes (e.g., cell division and differentiation) [8].

Lin et al. reported ratiometric fluorescent probes 22 and 23 for specific detection of RSH [62,63] (Fig. 6). Probe 22 could detect Cys over Hcy and GSH based on coumarin aldehyde [62]. In the absence of Cys, probe 22 displayed an emission band at 557 nm, while, upon treatment with Cys, it showed dramatic variation. There was a marked decrease of emission intensity at 557 nm and concurrently a dramatic enhancement of emission intensity at 487 nm, which made 22 suitable for imaging Cys in living cells.

Probe 23 was constructed for detecting intracellular RSH based on FRET from coumarin to porphyrin, as the coumarin emission spectra matched well with the porphyrin absorption spectra [63]. The addition of Cys elicited significant fluorescence enhancement at 459 nm.

Figure 12. Structures of cell-trappable fluorescent probes 42–50.
(emission wavelength of coumarin) but almost no changes in fluorescence intensity at 658 nm (emission wavelength of porphyrin). The emission ratio \(F_{459}/F_{658}\) showed a 60-fold enhancement from 0.21 in the absence of Cys to 12.1 in the presence of Cys (500 equiv). The emission ratios \(F_{459}/F_{658}\) exhibited excellent linearity with Cys in the range 1–600 \(\mu\)M. The LOD (S/N = 3) of the probe was determined to be 0.73 \(\mu\)M. The combination of the low LOD and the large fluorescence dynamic range indicated that it was suitable for ratiometric imaging and detecting RSH in HeLa cells.

Kim et al. reported a ratiometric two-photon fluorescent probe \(24\) for selective detection of mitochondrial thiols \([64]\). A disulfide group was selected as the thiol-reaction site, 6-(benzo[d]thiazol-2′-yl)-2-(N,N-dimethylamino) naphthalene (BTDAN) as the reporter and triphenylphosphonium salt (TPP) as the mitochondria-targeting site.

2.7. Challenges for ratiometric fluorescent probes

Based on self-calibration of fluorescence intensity to erase the potential origin inaccuracy of cell imaging, ratiometric fluorescent probes have been widely applied for imaging ISMRS \([22–26]\), which is a major advancement in developing fluorescent probes. However, there are still several challenges in the development of ratiometric fluorescent probes:

1. the sensitivity of ratiometric fluorescent probes in detecting ISMRS needs to be much higher;
the species (e.g., Fe\(^{2+}\), Cu\(^{2+}\), Cl\(^{-}\), O\(_2\), ONOO\(^{-}\)) that frequently cause quenching of fluorophores upon collision with the fluorescent probes are intractable to be detected by conventional fluorescent probes, and ratiometric fluorescent probes designed for detecting these species will be more necessary.
Future research on detecting ISMRS will benefit from these ratiometric fluorescent probes.

### 3. Organelle-localized ISMRS fluorescent probes

Genetically-encoded tags (e.g., GFP) are traditionally used to monitor ISMRS in situ. However, they can cause significant perturbations to protein structure, which would have an effect on imaging ISMRS [36]. Compared with this technique, SNAP-tag (SNAP-tag is derived from SNAP-transferase (AGT) and is labeled using O6-benzylguanine derivatives)-containing fluorescent probes and organelle-targetable small-molecule fluorescent probes that operate more conveniently offer an alternative methodology for designing organelle-localized probes and studying localized ISMRS fluxes [12,17,65–84]. SNAP-tag employs transfection using standard liposome-mediated gene-transfer techniques [12,65,67–69]. Organelle-targetable fluorescent probes can be achieved by incorporating or covalently modifying a unique bio-orthogonal chemical tag into a conventional fluorescent and luminescent probe. The bioorthogonal chemical tag is often as small as a single functional group that could accumulate in specific organelles (Fig. 7). For the visualization of local ISMRS signaling in specific organelles, several bioorthogonal chemical tags that could be incorporated into single fluorescent probes have been developed [17,66–84]. Recently, organelle-localized probes for ISMRS became very popular, some being particularly useful to assess homeostasis of thiol [64], Zn2+ [67,70,71], Ca2+ [68], Cu+ [72,81], H2O2 [69,74,80], O2− [76], O3− [77], hROS [78] fluxes, oxidative stress [73], temperature changes [79] and pH fluctuations [82–84] in different organelles.

#### 3.1. Strategies to design and to fabricate sub-cellular targetable fluorescent probes

ISMRS-responsive organelle-targeted probes could be selectively targeted to nucleus, mitochondria, endoplasmic reticulum and plasma membrane by fusion of SNAP-tag to the C-terminus of the histone H2B protein (SNAP-H2B), the C-terminus of cytochrome c oxidase subunit 8 (Cox8A), a signaling peptide for retention of the protein in endoplasmic reticulum (KDEL), the N-terminus of neurokinin-1 receptor (NK1R) and the C-terminus of 5HT3A serotonin-receptor-signaling sequence.

In 2008, Lippard et al. designed organelle-specific Zn2+ probe 25 that combined the Zn2+-sensitive sensor and AGT substrate benzylguanine [67] (Fig. 8). By fusion with human-1, 4-galactosyltransferase to direct AGT to the trans-Golgi cisternae and sub-unit VIII of cytochrome c oxidase to direct AGT to the mitochondrial matrix, these probes could separately monitor Zn2+ flux in Golgi apparatus and the mitochondria.

In 2009, Rios and Johnson et al. introduced a similar method to measure Ca2+ concentrations inside nuclei of living cells, based on linking Ca2+-sensitive dye Indo-1 to SNAP-tag fusion proteins [68].

In 2010, Chang et al. exploited the versatility of the SNAP-tag technology for site-specific protein labeling with the use of boronate-capped dyes 27 to visualize H2O2 selectively in different organelles [69]. These H2O2-responsive organelle-targeted reporters were selectively targeted to nucleus, mitochondria, endoplasmic reticulum and plasma membrane.

#### 3.2. Mitochondria-targetable fluorescent probes

Mitochondria is a conventional primary source of ROS in the course of metabolizing oxygen and releasing surplus energy in the form of heat through respiration. On the ETC in mitochondria, NADPH catalyzes the production of large variety of reactive oxidants, including ROS, RCS and free radicals. High levels of ROS can trigger cell death, whereas lower levels drive diverse and important cellular functions. To distinguish the variable concentration of ROS in mitochondria will be of paramount significance. When there is higher Zn2+ concentrations in living cells, O2− production increases, which results in mitochondrial dysfunction, formation of additional ONOO−, and amplification of the Zn2+ and NO signaling pathway, which lead to cell death. The fluorescent probe targeting the intracellular mitochondria is through the use of small chemical tags bearing lipophilic cations that are attracted to the inner mitochondrial membrane with negative potential. So far, such small chemical tags are:

1. TPP tag;
2. mitochondria-targeted peptides (MPPs); and,
3. environmentally sensitive fluorophores that can be specifically targeted to mitochondrial micro-environments (e.g., MitoTracker, rhodamine and cyanine dyes) [17,65,75].

The fluorescent probes incorporated with small chemical tags bearing lipophilic cations possess an overall positive charge, so they could accumulate in the mitochondria of living cells for a long time. Confocal microscopy experiments further validate that the targetable fluorescent probes accumulate within the mitochondria of living cells.

For detecting mitochondrial Zn2+ fluxes, 28 and 29 have been developed [70,71] (Fig. 9). In 2011, Kim et al. reported two-photon probe 28 for mitochondrial Zn2+ [70]. The probe showed a 7-fold enhancement of two-photon-excited fluorescence in response to Zn2+ without interference from other metal ions, allowing the detection of Zn2+ in a rat hippocampal slice at a depth of 100–200 μm through the use of TPM.
In 2011, Davidson and Zhu et al. developed a mitochondria-localizing Zn\(^{2+}\) probe \(30\) by introducing a TPP moiety to the side of diamino-substituted naphthalenediimide \(71\). Imaging of \(29\) in living TPM cells showed its good colocalization with mitochondria, which made it suitable for selectively detecting Zn\(^{2+}\) over other mitochondrial metal ions (e.g., Fe\(^{2+}\) and Cu\(^{2+}/\)Cu\(^{+}\)).

In 2011, Chang developed mitochondria-targeted fluorescent probe \(30\) for monitoring Cu\(^{+}\) homeostasis \(72\). Probe \(30\) was achieved by introducing a carboxylic-acid handle conjugated with a TPP tag into probe \(3\). Using probe \(30\) revealed that Cu\(^{-}\)-deficient SCO1 and SCO2 (two synthesizes of cytochrome c oxidase genes) patient cells prioritize mitochondrial copper homeostasis.

In 2007, Miyata et al. developed TEMPO derivative \(31\) that has a nitroxyl-radical moiety for measuring ESR, and fluorescein moiety for confirming distribution in cells with TPP moiety for evaluating oxidative stress in mitochondria \(73\).

In 2008, Chang et al. employed newly-developed mitochondria-targeted \(32\) by introducing the \(H_2O_2\)-cleavable boronate switch and a TPP tag into the rhodamine scaffold \(74\). Probe \(32\) featured a turn-on response for \(H_2O_2\) and could selectively detect subcellular changes in \(H_2O_2\) fluxes in the mitochondria of living cells.

Permeant cationic fluorescent probes (e.g., cyanine probes) can be selectively accumulated by the mitochondria of living cells \(75\). In 2011, our group developed cyanine probes \(33\) for imaging \(^1\)O\(_2\) and \(34\) for imaging \(O_3\) in the mitochondria of living cells \(76,77\). Probe \(33\) comprised cyanine as fluorophore and histidine (His) as \(^1\)O\(_2\)-selective modulator. To construct probe \(34\), we chose tricarbocyanine as NIR fluorescent dye and L-tryptophan (Trp) as an \(O_3\)-indicator based on a twisted intramolecular charge transfer (TICT) mechanism.

In 2007, Nagano et al. selected 4-aminophenyl aryl ether and 4-hydroxyphenyl aryl ether as selective modulators based on rhodamine dye to synthesize fluorescent probes \(35a\) and \(35b\) for selective detection of hROS in mitochondria of living cells \(78\). \(35a\) and \(35b\) reacted with intracellular hROS selectively over other ROS as due to the strong oxidation capacity of hROS.

3.3. Nuclear-targetable fluorescent probes

Though various fluorescent probes targeting to the mitochondria are extensively explored with an explicit mechanism, how to target other organelles (e.g., nuclear and lysosome) is still not definite and more studies are needed to design targetable fluorescent probes for other organelles. There are a few reports on nuclear-targetable fluorescent probes and lysosome-targetable fluorescent probes, which are only validated by confocal microscopy experiments. More studies need to elaborate further the mechanism of how the fluorescent probes accumulate within the corresponding organelles. As reported by Uchiyama et al., through a nano fluorescent polymeric thermometer \(36\) (Fig. 10), the distribution of intracellular temperatures in the nucleus and the centrosome of a COS7 cell differed and showed a significantly higher temperature than in the cytoplasm \(79\). As there are many cellular events in the nucleus (e.g., DNA replication, transcription and RNA processing) and its structural separation by the nuclear membrane that contribute to the temperature difference \(79\), ISMRS may also maintain different levels in the nucleus and other organelles.

In 2011, Chang et al. developed a nuclear-targetable fluorescent \(H_2O_2\) probe \(37\), which revealed a link between longevity-promoting sirtuin protein and enhanced regulation of nuclear ROS pools \(80\). Probe \(37\) selectively accumulated in the nuclei of a variety of mammalian cell lines and showed selective response to sub-cellular \(H_2O_2\) changes, which proved to be suitable for monitoring sirtuin-mediated oxidative stress responses. However, the molecular mechanism of \(37\) localized in the nucleus and the effects of temperature on the homeostasis of ISMRS remain unclear and deserve future investigation.

3.4. Lysosome-targetable fluorescent probes

The proton concentration within mammalian cells can vary in the range 10–1000 nM \(12\). Many biological events and processes can be monitored by measuring local intracellular fluctuations in the value of pH. The mechanism of fluorescent probes accumulating in acidic vesicles is through incorporation of chemical tags that tend to be reactive toward protons in the protonated form (e.g., anilines). They should be weak or non-fluorescent in the non-protonated form at pH 7.4 and become highly fluorescent in the protonated form under acidic conditions (pH < 6). However, limitations of the currently available pH probes include low sensitivity and excitation profiles in the ultraviolet region that can damage living samples and cause interfering autofluorescence from native cellular molecules. Also, very few acidic fluorescent probes are considered to be desirable for studying acidic organelles, which is a bottleneck in cell biological or medical studies.

Our group designed NIR fluorescent probe \(38\) for imaging and detecting Cu\(^{2+}\) (Fig. 11) \(81\). Probe \(38\) effectively avoided the fluorescence quenching for the paramagnetic nature of Cu\(^{2+}\) via its strong binding capability toward Cu\(^{2+}\) in living cells with high selectivity, high sensitivity, good photostability and the ability to work within the acidic pH range. It was interesting to find that synthesized fluorescent probe \(38\) tended to accumulate in lysosomes.

In 2009, Kobayashi et al. reported a series of acidic pH-sensitive fluorescence probes \(39\) based on BODIPY.
bearing hydrogen, methyl or ethyl substituents on the aniline nitrogen for selective molecular imaging of viable cancer cells [82]. They were constructed by targeting the monoclonal antibody into carboxylic groups on BODIPY with the human epidermal growth factor receptor type 2 (HER2). They could be internalized via the endosomal-lysosomal degradation pathway after binding to HER2. They were almost non-fluorescent in the extracellular environment (at pH 7.4) and became highly fluorescent in the protonated form under acidic conditions (pH < 6).

Ying et al. similarly synthesized cell-permeable BODIPY analogs 40 for selective labeling and monitoring pH changes of lysosomes in living cells [83]. Fluorescent imaging showed that probe 40 was suitable for lysosome labeling, and for non-invasive monitoring of lysosomal pH changes during physiological and pathological processes.

Gao et al. construed a series of nanoprobes 41 to target the acidic endosomal/lysosomal compartments. They were prepared based on the supramolecular self-assembly of ionizable block copolymer micelles containing tetramethyl rhodamine (TMR) as pH-insensitive reference dye [84]. The NPs can be selectively activated at various pH values, especially when in endocytic compartments (e.g., early endosomes or lysosomes in human cells).

### 3.5. Cell-trappable fluorescent probes

There is emerging evidence that improvement of cellular retention of fluorescent probes in organelles or in cytosol is a practical, promising strategy to improve sensitivity of fluorescent probes. A repertoire of cell-trappable ISMRS-sensitive fluorescent probes has therefore been developed and applied to cell imaging [85–94]. Typically, there are two approaches to increase cellular retention of fluorescent probes – ester functionalization [85–93] and polymer conjugation [94] – which are achieved through modification of carboxylic moieties to acetoxymethyl (AM) esters or conjugation to polymer. On entering into the cells, the ester groups are hydrolyzed by non-specific intracellular esterases. Cell-trappable ISMRS-sensitive fluorescent probes show superior intracellular retention, as negatively charged tetracarboxylates or polymer cannot penetrate cellular membranes, so the fluorescent probes are restricted to the cytosol and the nucleus, which, in turn, increase sensitivity and make them possible to visualize much lower concentrations of specific ISMRS. This advantage contributes to making it possible to perform longer observation in living cells.

In 1981, Tsien developed the first cell-trappable fluorescent probe 42 (Fig. 12) for Ca2+ by means of ester functionalization, and it had unusual specificity for Ca2+ over Mg2+, H+, Na+ and K+ [85]. Since that time, many efforts have been made to convert the probes into the corresponding ester derivatives or to conjugate probes to polymers.

In 2010, Lippard et al. developed an esterified ligand for Zn2+, which could be effectively trapped by cells [86]. Probe 43 was poorly emissive in the off-state but exhibit dramatic increases (10-fold) in fluorescence upon Zn2+ binding over other biologically relevant metal ions. It was cell-membrane-permeable until cleavage of its ester groups by intracellular esterases produced a negatively-charged acid form that could not cross the cell membrane.

In 2011, Taki et al. developed a cholesterol-conjugated fluorescence Zn2+ probe 44 capable of detecting extracellular Zn2+ in the vicinity of the plasma membrane [87]. In the absence of Zn2+, the fluorescence of 44 is nearly quenched (U < 0.005) because of the PET process and is enhanced (~45-fold) upon coordination with Zn2+ (U = 0.18). This result indicated that the cholesterol moiety strongly interacted with membrane lipids, resulting in the probes being located at the extracellular region because of the high polarity of the carboxyl group.

Fluorescent probe 45a was reported by Hempel et al. [88]. It was generated by conjugating diacetate ester to 2′,7′-dichlorodihydrofluorescein diacetate (CM-DCFH). After it was passively diffused into cells, the two acetate groups were cleaved by intracellular esterases to yield DCFH. To improve cellular retention of the oxidation product, a chloromethyl derivative of 45a, 5-(and 6-)chloromethyl-2′,7′- dichlorodihydrofluorescein diacetate (CM-DCFH DA) 45b, was subsequently prepared by Jackson et al. [89]. The chloromethyl group of 45b allowed for covalent binding to intracellular thiol components, resulting in retention of CM-DCF within the cell for longer time intervals. Accumulation of CM-DCF in cells can be measured on the basis of an increase in fluorescence at 530 nm on excitation at 488 nm.
Figure 14. Structures of reversible fluorescent probes 51–55 for detecting ISMRS.
In 2010, Chang et al. developed two new probes, 46 and 47, which took advantage of multiple masked carboxylates to increase cellular retention and hence sensitivity to low levels of H$_2$O$_2$ [90,91]. In their ester-protected forms, the 46 and 47 dyes are more lipophilic than their carboxylate counterparts and can readily enter cells. Once inside cells, the protecting groups are rapidly cleaved by intracellular esterases to produce their anionic carboxylate forms, which are effectively trapped within cells because they cannot pass back through the plasma membrane. The increased retention of 46 and 47 leads to their enhanced sensitivity to H$_2$O$_2$ through irreversible boronate-oxidation events.

In particular, probe 46 was utilized to interrogate the cellular mechanisms involved in trafficking H$_2$O$_2$ during growth-factor signaling. Certain classes of aquaporin water channels, the aquaglyceroporins and unorthodox aquaporins but not classic aquaporins, can enhance the uptake of extracellularly-produced H$_2$O$_2$ and regulate intracellular signal transduction. This work represented the first study revealing that aquaporins can mediate both H$_2$O$_2$ transport and signaling in mammalian cells and had broad implications for H$_2$O$_2$ biology in processes ranging from cell migration to wound repair [90].

In parallel work using 46 for imaging, the roles of H$_2$O$_2$ in the self-renewal of neural stem cells were explored. It was discovered that adult hippocampal progenitor cells (AHPs) required basal generation of H$_2$O$_2$ for their normal growth and proliferation in cell culture and in vivo and determined that the NOX2 enzyme and phosphatase PTEN are molecular sources and targets of H$_2$O$_2$, respectively. This study provided evidence that H$_2$O$_2$ is a physiological regulator in living organisms, demonstrating that controlled ROS levels are needed to maintain specific cell populations [91].

In 2009, Nagano et al. found that fluorescein-based fluorescent probes 48 and 49 containing the iminodiacetic acid group (IAG) showed superior intracellular retention, which could be utilized to increase the sensitivity of fluorescence probes [92]. Probe 48 was sensitive to NO and 49 gave selective response to hROS. They were successfully applied to cell imaging.

In 2010, Lippard et al. developed cell-trappable NO-sensitive probes 50a and 50b by incorporating an ester moiety and a dextran moiety into fluorescein-based symmetrical ligands [93,94]. Cu(II) complexes as NO-specific fluorescent probes have been illustrated in a previous report. Both 50a and 50b responded rapidly and selectively to NO over other ROS and RNS in mouse olfactory bulb slice. Considering the dynamic changes of ROS, RNS and pH, it is of paramount significance to design multi-functional fluorescent probes that could simultaneously monitor several metabolites [95].

3.6. Challenges for sub-cellular targetable fluorescent probes

Imaging the homeostasis of ISMRS by use of targetable fluorescent probes is critical for further research on cellular function. However, considering the complexity of the microenvironments in different organelles, several factors should be taken into account during design of targetable fluorescent probes:

(i) the temperature in mitochondria and nuclei is obviously higher than other organelles, which has a significant effect on fluorescence, so temperature-insensitive fluorescent probes are preferred;

(ii) the pH value in acidic organelles [e.g., lysosomes and endosomes (pH range 4.5–6.0)] is lower than in other organelles;

(iii) the relative level of ISMRS is different in various organelles (e.g., H$_2$O$_2$ and O$_2$$^\cdot$ are estimated to be in the ratio of around 100:1 in the mitochondrial matrix but around 1000:1 in the cytosol). The studies of such factors are still not explicit. Only if they are studied deeply could the complex mechanism on the role on ISMRS in organelles be fully unraveled. The perfect targetable fluorescent probes should be single-factor responsive, not multi-factor responsive, so as to assure a proper fluorescent signal. Future design will focus on the elaborate microenvironments of various organelles in living cells and environmentally-sensitive chemical tags that can be specifically targeted to sub-cellular microenvironments.

4. Reversible fluorescent probes for imaging of redox events in living cells

An important development of functional fluorescent probes in living cells would be the possibility to monitor the dynamic redox homeostasis by reversible fluorescent
Figure 16. Structures of multi-functional fluorescent probes 56–58.
probes. Redox homeostasis is critical for proper cellular function and metabolism. Changes to the intracellular redox environment would cause oxidative stress, which has been implicated in a variety of diseases, including cancer, ischemia-reperfusion injury, and neurodegeneration [96,97,100,109,119]. The redox status of the cell is primarily a consequence of the precise balance between the levels of ROS and reducing equivalents (e.g., organoselenium) and the thiol-disulfide interchange reaction [96,119]. A hallmark of oxidative stress is the abnormal ratio of GSH to its disulfide in cells [97,119].

Currently, four redox processes are used for designing reversible fluorescent probes: (1) the electron-loss and electron-capture process (e.g., ferrocene [98,99]); (2) dehydrogenation and hydrogenation reaction (e.g., fluorescein [100], tetrathiafulvalene [101,102], hydroquinone-benzoquinone switching [103–107], 2,2,6,6-tetramethyl-1-piperidin-oxyl [108,109], and NADH analogue [110–112]); (3) oxygenation-deoxidation reaction (e.g., oxidation of organoselenium [113,114] or tellurium [115,116]); and, (4) thiol-disulfide interchange reaction [117–119].

Modulating the fluorescence of reversible fluorescent probes is achieved by reversible binding with ISMRS along with the four redox processes (Fig. 13). Among them, several interesting small-molecule fluorescent probes 51–55 that can measure intracellular redox environments and related ISMRS have been developed [100,109,114,119,120] (Fig. 14).

Based on the dehydrogenation and hydrogenation reaction, Chang et al. devised fluorescent probe 51 for imaging reversible redox cycles in living cells by introducing disulfide into fluorescein [100]. Bottle et al. designed a novel profluorescent nitroxide 52 based on fluorescein as a sensitive probe for the cellular redox environment [109]. Probe 52 could sensitively quantify changes of intracellular redox status, especially in giving a selective response to in vitro O2•− over H2O2 and ‘OH.

Inspired by the principal modes of enzymatic ROS scavenging by the ascorbate-GSH cycle and the glutathione peroxidase (GPX) cycle, our group prepared NIR reversible fluorescent probe 53 for ONOO− and imaging of redox cycles in living cells [114].

Murthy et al. chose coumarin as fluorophore and conjugated a thiolate to its extended aromatic π system as a sensitive fluorescent probe 54 to measure the thiol-disulfide equilibrium of biological systems reversibly and ratiometrically [119]. The fluorescence emission of 54 and its disulfide are different after excitation at 372 nm and 488 nm, which allow for ratiometric imaging of the thiol-disulfide dynamics (dynamic GSH/GSSH ratios). Probe 54 was applied to monitor the reversible redox status of whole-cell lysates.

You et al. and Chen et al. developed Fe2+–selective fluorescent bioimaging probes 55 [120] by introducing the imidazolium-based ionic liquid pendants into the squaraine skeleton and controlling the aggregation behavior of squaraines by adjusting the alkyl chain attached to the imidazolium unit. In the absence of Fe2+, probe 55 was fluorescent. However, fluorescence disappeared upon the addition of both FeCl2 and H2O2 that generated ‘OH due to attack the ‘OH to the four-membered ring of squaraine. Moreover, the cells could light up renewably in 20 min after a fluorescence “turn-off” response. These imidazolium-anchored squaraines in combination with the Fenton Reaction could perform “naked-eye” detection of the Fe2+ over other metal ions in living cells.

In considering the reactive nature of ROS, RNS and RCS, the fluorescent probes must provide fast, selective and sensitive response to them. To give further reversible response to them, there are few fluorescent probes to meet this requirement, so the challenges in reversible fluorescent probes include: (1) developing fluorescent probes with more detection cycles and better biocompatibility; (2) design of highly specific fluorescent probes for only one species; and, (3) multi-functional probes that could simultaneously monitor several reversible cellular processes.

5. Multi-functional probes for simultaneous imaging of multiple components in living cells

Another important development of functional fluorescent probes in living cells would be the possibility to report on ISMRS concentrations simultaneously with different fluorescent probes for multi-modal or multi-parameter imaging. As far as the complex cellular environment is concerned, different fluorescent probes for multi-modal or multi-parameter imaging should be assembled into a molecular cargo [e.g., chemically-modified nanomaterials or fluorophores (e.g. coumarin and rhodamine dyes)], which are collectively termed multi-functional probes (Fig. 15).

To build such multi-functional probes is a great need for chemists and biologists. As ISMRS are involved in the dynamic changes, some of them, especially ROS and RNS, are very reactive species that have short lifetimes of ~ns [1–4]. Intracellular environmentally-sensitive parameters, including intracellular viscosity, protease activity and temperature, are closely connected with transportation and diffusion of reactive metabolites (e.g., ROS and RNS) [79]. Changes in them at cellular level have a great effect on diseases and pathologies, so every particular metabolite of ISMRS and intracellular environmentally-sensitive parameters need to be
monitored directly near the sites of production and action (in situ).

Recently, Lin et al. chose phenylenediamine as the reaction site of NO, the boronate group as the H2O2-responsive site and 7-hydroxycoumarin and rhodamine dyes as fluorophores to synthesize multi-functional imaging probe 56 that could simultaneously report intracellular H2O2, NO, and H2O2/NO with a distinct fluorescence signal pattern of blue–black–black, black–black–red, and black–red–red, respectively [121] (Fig. 16).

Nanostructures provide a broad platform to design multi-functional fluorescent probes due to their prominent engineerability [122–131]. Nanomaterials with unique properties (e.g., polymers, carbon nanotubes, magnetic NPs and noble-metal NPs) have shown great promise for cancer therapy.

So far, several nanoprobes have been utilized for multi-functional detection and therapeutic application in biomedical imaging. Liu et al. fabricated dual-functional nanoprobe 57 based on hydrophilic block copolymers for detecting changes in temperature and Zn2+ [122]. Nanoprobe 57 was achieved through polymerization of a thermo-responsive block by incorporating quinoline-based Zn2+-responsive fluorescent probe (ZQMA) into the polymer. At low temperature, nanoprobe 57 could selectively bind Zn2+ over other metal ions, resulting in the prominent fluorescence enhancement. Fluorescent images of 57 in HeLa cells promoted the investigation of the effects by thermo-induced micellization and detection conditions of intracellular Zn2+ fluxes.

Our group also designed several multi-functional platform and fluorescent probes with simultaneous targeting, therapeutic and imaging functions for studying the inter-conversion of ROS and therapeutic applications [123–131]. Bifunctional Au-Fe3O3 NPs exhibited simultaneous targeting, therapeutic and imaging functions in cancer [127]. The bi-photosensitizer molecular beacon could report the specific DNA/RNA with high selectivity and generate 1O2 effectively in breast-cancer cells, but not in normal cells, which holds great promise for cancer diagnosis and therapy [128].

In 2011, Chang et al. selected acetyl-capped G5 PAMAM dendrimers functionalized with boronate-caged Peroxyfluor-1 fluorophores for H2O2 detection and seminapthorhodafluor dyes for pH sensing as the platform to construct 58 for simultaneously detecting intracellular pH and H2O2 [132]. Probe 58 could give a simultaneous response to changes in H2O2 and pH by using different excitations resulting in different emissions, which allowed for selective discrimination between H2O2 and pH changes in live RAW 264.7 macrophage cells.

The ultimate development of functional fluorescent probes in living cells would be the possibility to design multi-functional probes to image simultaneously ISMRS and related intracellular environmentally-sensitive parameters. We note that, during the assembly of multi-functional probes, the differences in the structures of the fluorescent probes for cations, ROS, RNS, RCS and RSS should be taken into account. These fluorescent probes usually consist of a selective signaling moiety and fluorophores.

For metal-responsive fluorescent probes, the majority of selective signaling moieties are amine-, ether- or thioether-rich receptors (e.g., dipicolylamine is frequently used as receptor for Zn2+). Activation or deactivation fluorescence is controlled by inhibition of PET by coordination of metals, which is a commonly employed signaling mechanism for fluorescence enhancement.

For proton-responsive fluorescent probes, the signaling moieties are often anilines. The fluorescent probes should be weak or non-fluorescent in the non-protonated form but become highly fluorescent in the protonated form under acidic conditions.

Different from metals and protons, ROS, RNS, RCS and RSS are relatively more reactive. For selectively detecting them, the signaling moiety is often elaborately designed to give a fast, highly selective response.

6. Summary and prospects

The visualization of various ISMRS implicated in diverse cellular processes requires the use of spectroscopically distinguishable fluorescent imaging reporters. Functional fluorescent and luminescent probes, including ratio fluorescent and luminescent probes, organelle-targetable fluorescent probes, reversible fluorescent probes, multi-functional probes and corresponding nanoprobes substantially improve the performance of fluorescent imaging reporters, which have an advantage over conventional fluorescent probes.

Furthermore, RSS are the emerging new species that were recently detected in living cells [133–137]. ISMRS homeostasis and signaling are important in the ageing process and associated diseases, but their exact roles remain insufficiently understood.

Compared with conventional fluorescent probes, functional probes have the advantages of multi-functionality, precision and enormous flexibility, allowing for the integration of multi-modal reporting fluorescent signals. Conclusively, imaging of intracellular ISMRS utilizing functional small-molecule fluorescent probes and nanoprobes are among the trends in development of imaging probes. This new avenue to study the inter-conversion of ISMRS holds the promise of elucidating the biological roles of ISMRS in signal transduction and regulating cellular function.

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References
